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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES

(57) Abstract

A therapeutic composition for inhibiting the function of a target polynucleotide sequence in a mammalian cell includes an agent that provides to a mammalian cell an at least partially double-stranded RNA molecule comprising a polynucleotide sequence of at least about 200 nucleotides in length, said polynucleotide sequence being substantially homologous to a target polynucleotide sequence. This RNA molecule desirably does not produce a functional protein. The agents useful in the composition can be RNA molecules made by enzymatic synthetic methods or chemical synthetic methods *in vitro*; or made in recombinant cultures of microorganisms and isolated therefrom, or alternatively, can be capable of generating the desired RNA molecule *in vivo* after delivery to the mammalian cell. In methods of treatment of prophylaxis of virus infections, other pathogenic infections or certain cancers, these compositions are administered in amounts effective to reduce or inhibit the function of the target polynucleotide sequence, which can be of pathogenic origin or produced in response to a tumor or other cancer, among other sources.

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METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES

Field of the Invention

The present invention relates to polynucleotide compositions which have an inhibitory or other regulatory effect upon the function of certain target polynucleotide sequences present in a mammalian cell, and for methods of using the compositions in therapeutic, prophylactic, diagnostic and research methods.

Background of the Invention

Polynucleotide compositions have been described for pharmaceutical uses, primarily for treatment or prophylaxis of disease in mammals, as well as in research in such fields. Specifically a great deal of activity presently surrounds the use of polynucleotide compositions in the treatment of pathogenic extracellular and intracellular infections, such as viral, bacterial, fungal infections, and the like. As one example, DNA vaccines are described to deliver to a mammalian cell *in vivo* an agent which combats a pathogen by harnessing the mammalian immune system. Thus, such vaccines are designed to express, for example, a viral protein or polypeptide, and elicit a humoral or cellular immune response upon challenge by the infective agent. Gene therapy vectors, on the other hand, are polynucleotide compositions generally designed to deliver to a mammalian cell a protein which is either not expressed, expressed improperly or underexpressed in a mammal. Such vectors frequently must address species specific immune responses to the those polynucleotide sequences that are recognized as antigenic or which evoke an unwanted cellular immune response.

Still other therapeutic uses of polynucleotide compositions are for the delivery of missing or underexpressed proteins to a diseased mammalian patient. Furthermore, polynucleotides are useful themselves as *in vivo* reagents, in diagnostic/imaging protocols, as reagents in gene therapy, in antisense protocols and in vaccine applications or otherwise as pharmaceuticals used to treat or prevent a variety of ailments such as genetic defects, infectious diseases, cancer, and autoimmune diseases.

Polynucleotides are also useful as *in vitro* reagents in assays such as biological research assays, medical, diagnostic and screening assays and contamination detection assays.

A host of problems well-known to the art has prevented the numerous polynucleotide compositions from becoming widely accepted as useful pharmaceutics. Thus, there are few such DNA vaccines or therapeutics which have yet been accepted by the medical community for the treatment of disease in mammals.

Phenomena have been observed in plants and nematodes that are mediated by polynucleotide compositions, and are referred to as post-transcriptional gene silencing and transcriptional silencing. This phenomenon demonstrates that transfection or infection of a plant, nematode or Drosophila with a virus, viroid, plasmid or RNA expressing a polynucleotide sequence having some homology to a regulatory element, such as a promoter or a native gene or a portion thereof already expressed in that cell, can result in the permanent inhibition of expression of both the endogenous regulatory element or gene and the exogenous sequence. This silencing effect was shown to be gene specific. See, for example, L. Timmons and A. Fire, Nature, 395:354 (Oct. 29, 1998); A. Fire *et al*, Nature, 391:806-810 (Feb. 19, 1998); and R. Jorgensen *et al*, Science, 279:1486-1487 (March 6, 1998)]. A DNA plasmid encoding a full-length pro-alpha 1 collagen gene was transiently transfected into a rodent fibroblast tissue cell line and a "silencing" effect on the native collagen gene and the transiently expressed gene observed [Bahramian and Zarbl, Mol. Cell. Biol., 19(1):274-283 (Jan. 1999)].

See, also, International Patent Application No. WO98/05770, published February 12, 1998, which relates to gene inhibition by use of an antisense RNA with secondary structures, and/or in combination with double stranded RNAse. International Patent Application No. WO99/53050, published October 21, 1999, also relates to reducing phenotypic expression of a nucleic acid, particularly in plant cells, by introducing chimeric genes encoding sense and anti-sense RNA molecules.

There exists a need in the art for polynucleotide compositions and methods of using same to inhibit the function of polynucleotide sequences which are disease-

causing in mammals, such as polynucleotide sequences essential for the replication of viruses and other intracellular pathogens in mammalian cells, or sequences of extracellular mammalian pathogens, or sequences of tumor antigens which mediate the spread of cancer in a mammal, and the like, without adversely affecting essential gene sequences in the mammal.

Summary of the Invention

In one aspect, the invention provides a composition for inhibiting the function of a target polynucleotide sequence in a mammalian cell. The composition comprises an agent that provides to a mammalian cell an at least partially double-stranded RNA molecule comprising a polynucleotide sequence of at least about 200 nucleotides in length. The polynucleotide sequence is substantially homologous to the target polynucleotide sequence, which can be a polynucleotide sequence, e.g., of a virus or other intracellular pathogen, a polynucleotide sequence of a cancer antigen or of an essential tumorigenic regulatory sequence, a polynucleotide sequence of an extracellular pathogen present in a mammal, or any other polynucleotide sequence which is desired to be "turned off" in a cell. This RNA molecule preferably does not produce a functional protein. This RNA molecule is preferably substantially non-homologous to naturally-occurring, essential mammalian polynucleotide sequences. In one embodiment, the agent of this composition is an RNA molecule made by enzymatic synthetic methods or chemical synthetic methods *in vitro*. In another embodiment, the RNA molecule may be generated in a recombinant culture, e.g., bacterial cells, isolated therefrom, and used in the methods discussed below. In another embodiment the agent of this composition generates the RNA molecule *in vivo* after delivery to the mammalian cell.

In another aspect, the invention provides a pharmaceutical composition comprising one or more of the compositions described immediately above and specifically hereinbelow, and an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier. Such compositions are useful for treating intracellular pathogenic infections, such as viruses. Other such

compositions are useful for treating certain cancers. Other such compositions are useful for treating certain extracellular pathogens. Still other such compositions are useful for treating any disease or disorder wherein inhibiting the function of a polynucleotide sequence in a mammal is desirable for therapy or vaccine use.

In still another aspect, the invention provides a method for treating a viral infection in a mammal by administering to the mammal one or more of the above-described compositions wherein the target polynucleotide is a virus polynucleotide sequence necessary for replication and/or pathogenesis of the virus in an infected mammalian cell, along with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier. This composition is administered in an amount effective to reduce or inhibit the function of the viral sequence in the cells of the mammal.

In yet a further aspect, the invention provides a method for preventing a viral infection in a mammal by administering to the mammal one or more of the above-described compositions wherein the target polynucleotide is a virus polynucleotide sequence necessary for replication and/or pathogenesis of the virus in an infected mammalian cell, with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier. This composition is administered in an amount effective to reduce or inhibit the function of the viral sequence upon subsequent introduction of the virus into the mammalian cells.

In still another aspect, the invention provides a method for treatment or prophylaxis of a virally induced cancer in a mammal by administering to the mammal one or more of the above described compositions in which the target polynucleotide is a sequence encoding a tumor antigen or functional fragment thereof or a regulatory sequence, which sequence function is required for the maintenance of the tumor in the mammal. The compositions can contain an optional second agent that facilitates polynucleotide uptake in a cell, and a pharmaceutically acceptable carrier. The composition is administered in an amount effective to reduce or inhibit the function of the tumor-maintaining sequence in the mammal.

In another aspect, the invention provides a method for the treatment or prophylaxis of infection of a mammal by an intracellular pathogen. The mammal is administered one or more of the compositions herein described wherein the target polynucleotide is a polynucleotide sequence of the intracellular pathogen necessary for replication and/or pathogenesis of the pathogen in an infected mammalian cell. The composition is administered with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier, in an amount effective to reduce or inhibit the function of the sequence in the mammal.

In another aspect, the invention provides a method for the treatment or prophylaxis of infection of a mammal by an extracellular mammalian pathogen. The mammal is administered one or more of the compositions herein described wherein the target polynucleotide is a polynucleotide sequence of the extracellular pathogen necessary for replication and/or pathogenesis of the pathogen in an infected mammal. The composition is administered in a pharmaceutically acceptable carrier, in an amount effective to reduce or inhibit the function of the sequence in the mammal. It may be administered with with an optional second agent that facilitates polynucleotide uptake by the pathogenic cell.

In still another aspect, the invention provides a method of treatment or prophylaxis of cancer in a mammal. The mammal is administered one or more of the above-described compositions, wherein the target polynucleotide is a polynucleotide sequence of an abnormal cancer-causing gene or non-expressed regulatory sequence in a mammal, which also possesses a normal copy of the gene or regulatory sequence. According to this aspect, the differences between the abnormal sequence and the normal sequence are differences in polynucleotides. The composition is administered with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier, and in an amount effective to reduce or inhibit the function of the abnormal sequence in the mammal.

In yet a further aspect, the invention involves a method for treating a disease or disorder in a mammal comprising administering to the mammal having a disease or disorder characterized by expression of polynucleotide product not found in a healthy

In still another embodiment, the RNA molecule polynucleotide sequence lacks a cap structure. In yet a further embodiment, the RNA molecule has no signals for protein synthesis. In still another embodiment, the RNA molecule contains no coding sequence or a functionally inoperative coding sequence. In still another embodiment, the RNA sequence can be punctuated with intronic sequences. In yet a further embodiment, a hairpin sequence can be placed before the native initiation codon, if present. In still another embodiment, the RNA molecule can be an RNA/DNA hybrid as described above. All such embodiments can be designed by resort to the known teachings of, e.g., such texts as cited below.

The following are various specific embodiments that may be used to achieve polynucleotide inhibition as described herein. It should be recognized that the various RNA (and RNA/DNA hybrid) structures described below may be used singly or in any combination of two or more, e.g., a lariat (sense or antisense) and/or a complementary circular and/or linear molecule. The antisense lariat or circle structures may also be used alone. Furthermore, these structures may include regions of self complementarity (e.g., tandem sense and antisense sequences) as well as additional antisense sequences relative to a desired target. Throughout this document the term "antisense" is used to mean complementary to and capable of hybridizing with any mRNA.

In one embodiment, polynucleotides in the form of "lariats" may be utilized. Lariats contain a 2'-5' phosphodiester linkage as opposed to the usual 3'-5' linkage. Such structures are formed in splicing reactions catalyzed by spliceosomes and self-cleaving ribozymes. These structures are either intermediates or by-products of splicing reactions. They can be prepared *in vivo* through expression (transcription) in a cell or prepared *in vitro*. Lariats form when a free 5' phosphoryl group of either a ribose or deoxyribose becomes linked to the 2' -OH of a ribose in a loop back fashion. The lariats may contain 10 or more nucleotides in the loop or may be a complete circle, with the loop back linkage in each case being 2'-5'. A lariat linking the terminal nucleotides produces a circle-like structure. The loops and/or the stem can contain either the sense and the antisense sequences in tandem in a single molecule, or

each single lariat contains either a sense or an antisense sequence. The lariats that contain sense and antisense in separate molecules may be administered together as a double-stranded form or the antisense lariat may be used singly to form a double strand with the mRNA in the cell. Lariats may be RNA or a DNA hybrid, with the 2'-5' linkage effected through the 2' –OH of the RNA portion of the hybrid [Rees C and Song Q. Nucl. Acid Res., 27, 2672-2681 (1999); Dame E *et al*, Biochemistry, 38, 3157-3167, 1999; Clement J.Q. *et al*, RNA, 5, 206-220, 1999; Block T and Hill J. J. Neurovirol., 3, 313-321, 1997; Schindewolf CA and Domdey H., Nucl. Acid Res., 23, 1133-1139 (1995)].

In another embodiment, a circular RNA (or circular RNA-DNA hybrid) can be generated through a 2'-5' or a 3'-5' linkage of the terminii. These may be generated enzymatically through RNA ligase reactions using a splinter to bring the ends in proximity *in vitro*, or through the use of self splicing ribozymes (*in vivo* and *in vitro*). The desired inhibition may be achieved by providing one or more RNA circles, made *in vitro* or expressed *in vivo*, including single circles with or without self complementarity, as well as double stranded circular RNA (both sense and antisense strands relative to the target polynucleotide), or two circles of single-stranded RNA which have regions of complementarity to each other as well as one having complementarity to a target.

Another embodiment utilizes single RNA (or RNA-DNA hybrid) antisense circles (circular RNA without self complementarity which is complementary to the target mRNA). Still another embodiment utilizes RNA-DNA circles or a circular DNA molecule complementary to a target mRNA molecule. Single circles with tandem sense and antisense sequences (in any order) which have complementarity to a target message may be used as the composition which inhibits the function of the target sequence. It may be preferred to use circular molecules having such self-complementary sequences which may form rod-like sections, as well as additional antisense sequences to the target [Schindewolf CA and Domdey H. Nucl. Acid Res., 23, 1133-1139 (1995); Rees C and Song Q., Nucl. Acid Res., 27, 2672-2681 (1999); Block T and Hill J., J. Neurovirol., 3, 313-321(1997)].

In yet a further embodiment, the composition which inhibits the target sequence is a capped linear RNA. Whether the dsRNA is formed *in vitro* or *in vivo*, either one or both strands may be capped. In circumstances where cytoplasmic expression would not ordinarily result in capping of the RNA molecule, capping may be accomplished by various means including use of a capping enzyme, such as a vaccinia capping enzyme or an alphavirus capping enzyme. A capped antisense molecule may be used to achieve the desired post transcriptional silencing of the target gene. Capped RNA may be prepared *in vitro* or *in vivo*. RNA made in the nucleus by RNA polII ordinarily is capped. Cytoplasmically expressed RNA may or may not be capped. Capping can be achieved by expressing capping enzymes of cytoplasmic viruses. Either both capped or one capped and one uncapped or both uncapped RNA or RNA-DNA hybrid sequences may be used in these compositions. Capped or uncapped antisense molecule may be used, singly or in any combination with polynucleotide structures described herein.

The RNA molecule according to this invention may be delivered to the mammalian or extracellular pathogen present in the mammalian cell in the composition as an RNA molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made *in vitro* by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1-882274-57-1.

Alternatively these molecules may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu *et al*, Nucl. Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin *et al*, Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby *et al*, Nucl. Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix *et al*, Nucl. Acids Res., 17(18):7381-93 (1989); S.H. Chou *et al*, Biochem., 28(6):2422-35 (Mar. 1989); O. Odai *et al*, Nucl. Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin *et al*, Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun *et al*, RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang *et al*, Nucl. Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov *et al*,

Nucl. Acids Res., 26 (18):4160-7 (Sept. 1998); M. Kadokura *et al*, Nucl. Acids Symp Ser, 37:77-8 (1997); A. Davison *et al*, Biomed. Pept. Proteins. Nucl. Acids, 2(1):1-6 (1996); and A. V. Mudrakovskaia *et al*, Bioorg. Khim., 17(6):819-22 (Jun. 1991)].

Still alternatively, the RNA molecule of this invention can be made in a recombinant microorganism, e.g., bacteria and yeast or in a recombinant host cell, e.g., mammalian cells, and isolated from the cultures thereof by conventional techniques. See, e.g., the techniques described in Sambrook et al, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, which is exemplary of laboratory manuals that detail these techniques, and the techniques described in US Patent Nos. 5,824,538; 5,877,159 and 65,643,771, incorporated herein by reference.

Such RNA molecules prepared or synthesized *in vitro* may be directly delivered to the mammalian cell or to the mammal as they are made *in vitro*. The references above provide one of skill in the art with the techniques necessary to produce any of the following specific embodiments, given the teachings provided herein. Therefore, in one embodiment, the "agent" of the composition is a duplex (i.e., it is made up of two strands), either complete or partially double stranded RNA. In another embodiment, the agent is a single stranded RNA sense strand. In another embodiment, the agent of the composition is a single stranded RNA anti-sense strand. Preferably the single stranded RNA sense or anti-sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti-sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti-sense strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double-stranded once it is in the cell, or *in vitro* during its synthesis. Still another embodiment of this invention is an RNA/DNA hybrid as described above.

Still another embodiment of the synthetic RNA molecule is a circular RNA molecule that optionally forms a rod structure [see, e.g., K-S. Wang *et al*, Nature, 323:508-514 (1986)] or is partially double-stranded, and can be prepared according to the techniques described in S. Wang *et al*, Nucl. Acids Res., 22(12):2326-33 (June 1994); Y. Matsumoto *et al*, Proc. Natl. Acad. Sci., USA, 87(19)7628-32 (Oct. 1990); Proc. Natl. Acad. Sci., USA, 91(8):3117-21 (Apr. 1994); M. Tsagris *et al*, Nucl. Acids Res., 19(7):1605-12 (Apr. 1991); S. Braun *et al*, Nucl. Acids Res., 24(21):4152-7 (Nov. 1996); Z. Pasman *et al*, RNA, 2(6):603-10 (Jun. 1996); P. G. Zaphiropoulos, Proc. Natl. Acad. Sci., USA, 93(13):6536-41 (Jun. 1996); D. Beaudry *et al*, Nucl. Acids Res., 23(15):3064-6 (Aug. 1995), all incorporated herein by reference. Still another agent is a double-stranded molecule comprised of RNA and DNA present on separate strands, or interspersed on the same strand.

Alternatively, the RNA molecule may be formed *in vivo* and thus delivered by a "delivery agent" which generates such a partially double-stranded RNA molecule *in vivo* after delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule "encoding" one of the above-described RNA molecules. The DNA agent provides the nucleotide sequence which is transcribed within the cell to become a double stranded RNA. In another embodiment, the DNA sequence provides a deoxyribonucleotide sequence which within the cell is transcribed into the above-described single stranded RNA sense or anti-sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double-stranded. Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above-described circular RNA molecule that optionally forms a rod structure or partial double strand *in vivo*. The DNA molecule may also provide for the in vivo production of an RNA/DNA hybrid as

described above, or a duplex containing one RNA strand and one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.

A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above-described RNA molecules, can be a DNA single stranded or double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein *in vitro* or *in vivo* may containing sequences under the control of any RNA polymerase, including mitochondrial RNA polymerase, RNA polI, RNA polII, and RNA polIII. These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondrial polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages *in vivo*. RNA pol I, RNA pol II, and RNA pol III transcripts may also be generated *in vivo*. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. The DNA vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA polI, II, or polIII, or viral, bacterial or bacteriophage promoters along with the cognate polymerases). Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts to avoid degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages. Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids. Thus, one exemplary vector is a single or double-stranded phage vector. Another exemplary vector is a single or double-stranded RNA or DNA viral vector. Such

and 65,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited reference, including, without limitation, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus*.

Still other delivery agents for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required RNA polynucleotide sequence discussed above. Such viruses may be designed similarly to recombinant viruses presently used to deliver genes to cells for gene therapy and the like, but preferably do not have the ability to express a protein or functional fragment of a protein. Among useful viruses or viral sequences which may be manipulated to provide the required RNA molecule to the mammalian cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno-associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses, papova viruses (such as SV40), poliovirus, pseudorabies viruses, retroviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viral delivery agents may be designed by applying conventional techniques such as described in M. Di Nocola *et al*, Cancer Gene Ther., 5(6):350-6 (1998), among others, with the teachings of the present invention.

Another delivery agent for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated donor cells which have been transfected or infected *in vitro* with a synthetic RNA molecule or a DNA delivery molecule or a delivery recombinant virus as described above. These donor cells may then be administered to the mammal, as described in detail below, to stimulate the mechanism in the mammal which mediates this inhibitory effect. These donor cells are desirably mammalian cells, such as C127, 3T3, CHO, HeLa, human kidney 293, BHK cell lines, and COS-7 cells, and preferably are of the same mammalian species as the mammalian recipient. Such donor cells can be made using techniques similar to those described in, e.g., Emerich

et al, J. Neurosci., 16: 5168-81 (1996). Even more preferred, the donor cells may be harvested from the specific mammal to be treated and made into donor cells by *ex vivo* manipulation, akin to adoptive transfer techniques, such as those described in D. B. Kohn *et al*, Nature Med., 4(7):775-80 (1998). Donor cells may also be from non-mammalian species, if desired.

Finally, the composition of this invention can also include one or more of the selected agents which are described above. The composition can contain a mixture of synthetic RNA molecules described above, synthetic DNA delivery molecules described above, and any of the other delivery agents described above, such as recombinant bacteria, cells, and viruses. The identity of the composition mixture may be readily selected by one of skill in the art.

B. Pharmaceutical (Therapeutic or Prophylactic) Compositions of the Invention

The compositions of this invention for pharmaceutical use desirably contain the synthetic RNA molecule as described above or the agent which provides that RNA molecule to the mammalian cell *in vivo* in a pharmaceutically acceptable carrier, with additional optional components for pharmaceutical delivery. The specific formulation of the pharmaceutical composition depends upon the form of the agent delivering the RNA molecule.

Suitable pharmaceutically acceptable carriers facilitate administration of the polynucleotide compositions of this invention, but are physiologically inert and/or nonharmful. Carriers may be selected by one of skill in the art. Such carriers include but are not limited to, sterile saline, phosphate, buffered saline, dextrose, sterilized water, glycerol, ethanol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, olive oil, sesame oil, and water and combinations thereof. Additionally, the carrier or diluent may include a time delay material, such as glycerol monostearate or glycerol distearate alone or with a wax. In addition, slow release polymer formulations can be used. The formulation should suit not only the form of

the delivery agent, but also the mode of administration. Selection of an appropriate carrier in accordance with the mode of administration is routinely performed by those skilled in the art.

Where the composition contains the synthetic RNA molecule or where the agent is another polynucleotide, such as, a DNA molecule, plasmid, viral vector, or recombinant virus, or multiple copies of the polynucleotide or different polynucleotides, etc., as described above, the composition may desirably be formulated as "naked" polynucleotide with only a carrier. Alternatively, such compositions desirably contain optional polynucleotide facilitating agents or "co-agents", such as a local anaesthetic, a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation such as polylysine, a branched, three-dimensional polycation such as a dendrimer, a carbohydrate, a cationic amphiphile, a detergent, a benzylammonium surfactant, or another compound that facilitates polynucleotide transfer to cells. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U. S. Patent Nos. 5,593,972; 5,703,055; 5,739,118; 5,837,533 and International Patent Application No. WO96/10038, published April 4, 1996; and International Patent Application No WO94/16737, published August 8, 1994, which are each incorporated herein by reference.

When the facilitating agent used is a local anesthetic, preferably bupivacaine, an amount of from about 0.1 weight percent to about 1.0 weight percent based on the total weight of the polynucleotide composition is preferred. See, also, International Patent Application No. PCT/US98/22841, which teaches the incorporation of benzylammonium surfactants as co-agents, administered in an amount of between about 0.001-0.03 weight %, the teaching of which is hereby incorporated by reference.

Where the delivery agent of the composition is other than a polynucleotide composition, e.g., is a transfected donor cell or a bacterium as described above, the composition may also contain other additional agents, such as those discussed in US Patents No. 5,824,538; 5,643,771; 5,877,159, incorporated herein by reference.

Still additional components that may be present in any of the compositions are, adjuvants, preservatives, chemical stabilizers, or other antigenic proteins. Typically, stabilizers, adjuvants, and preservatives are optimized to determine the best formulation for efficacy in the target human or animal. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable stabilizing ingredients which may be used include, for example, casamino acids, sucrose, gelatin, phenol red, N-Z amine, monopotassium diphosphate, lactose, lactalbumin hydrolysate, and dried milk. A conventional adjuvant is used to attract leukocytes or enhance an immune response. Such adjuvants include, among others, Ribi, mineral oil and water, aluminum hydroxide, Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic plyois, muramyl dipeptide, killed *Bordetella*, and saponins, such as Quil A.

In addition, other agents which may function as transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with the composition of this invention, include growth factors, cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), colony stimulating factors, such as G-CSF, GM-CSF, tumor necrosis factor (TNF), epidermal growth factor (EGF), and the interleukins, such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12. Further, fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicular complexes such as squalene and squalene, and hyaluronic acid may also be used administered in conjunction with the compositions of the invention.

The pharmaceutical compositions may also contain other additives suitable for the selected mode of administration of the composition. Thus, these compositions can contain additives suitable for administration via any conventional route of administration, including without limitation, parenteral administration, intraperitoneal administration, intravenous administration, intramuscular administration, subcutaneous administration, intradermal administration, oral administration, topical

administration, intranasal administration, intra-pulmonary administration, rectal administration, vaginal administration, and the like. All such routes are suitable for administration of these compositions, and may be selected depending on the agent used, patient and condition treated, and similar factors by an attending physician.

The composition of the invention may also involve lyophilized polynucleotides, which can be used with other pharmaceutically acceptable excipients for developing powder, liquid or suspension dosage forms, including those for intranasal or pulmonary applications. See, e.g., Remington: The Science and Practice of Pharmacy, Vol. 2, 19^{th} edition (1995), e.g., Chapter 95 Aerosols; and International Patent Application No. PCT/US99/05547, the teachings of which are hereby incorporated by reference. Routes of administration for these compositions may be combined, if desired, or adjusted.

In some preferred embodiments, the pharmaceutical compositions of the invention are prepared for administration to mammalian subjects in the form of, for example, liquids, powders, aerosols, tablets, capsules, enteric coated tablets or capsules, or suppositories.

The compositions of the present invention, when used as pharmaceutical compositions, can comprise about 1 ng to about 20 mgs of polynucleotide molecules as the delivery agent of the compositions, e.g., the synthetic RNA molecules or the delivery agents which can be DNA molecules, plasmids, viral vectors, recombinant viruses, and mixtures thereof. In some preferred embodiments, the compositions contain about 10 ng to about 10 mgs of polynucleotide sequences. In other embodiments, the pharmaceutical compositions contain about 0.1 to about 500 µg polynucleotide sequences. In some preferred embodiments, the compositions contain about 1 to about 350 µg polynucleotide sequences. In still other preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 µg of the polynucleotide sequences. In some preferred embodiments, the vaccines and therapeutics contain about 100 µg of the polynucleotide sequences.

The compositions of the present invention in which the delivery agents are donor cells or bacterium can be delivered in dosages of between about 1 cell to about

10^7 cells/dose. Similarly, where the delivery agent is a live recombinant virus, a suitable vector-based composition contains between $1x10^2$ pfu to $1x10^{12}$ pfu per dose.

Given the teachings of this invention, and the observed capacity of the inhibitory effect of the methods and compositions of this invention to be propagated to more cells than the cells transfected or infected with the composition of this invention, it is likely that suitable dosage adjustments can be made downwards from the above-noted dosages. Thus, the above dosage ranges are guidelines only. In general, the pharmaceutical compositions are administered in an amount effective to inhibit or reduce the function of the target polynucleotide sequence for treatment or prophylaxis of the diseases, disorders or infections for which such target functions are necessary for further propagation of the disease or causative agent of the disease. The amount of the pharmaceutical composition in a dosage unit employed is determined empirically, based on the response of cells *in vitro* and response of experimental animals to the compositions of this invention. Optimum dosage is determined by standard methods for each treatment modality and indication. Thus the dose, timing, route of administration, and need for readministration of these compositions may be determined by one of skill in the art, taking into account the condition being treated, its severity, complicating conditions, and such factors as the age, and physical condition of the mammalian subject, the employment of other active compounds, and the like.

C. *Therapeutic and Prophylactic Methods of the Invention*

The methods of this invention can employ the compositions described in detail above, and possibly other polynucleotide sequences currently used in the art (e.g., polynucleotide molecules which do encode proteins, whether functional or non-functional, or known RNA catalytic sequences, such as ribozymes) which can provide partially double stranded RNA molecules to a mammalian cell. It is anticipated, however, that the efficiency of these methods is enhanced by the use of RNA molecules which do not produce protein. These methods reduce or inhibit the function of a target polynucleotide sequence(s) in a mammal or in the cell of a

is administered with an polynucleotide uptake enhancer or facilitator and an optional pharmaceutically acceptable carrier. The amount or dosage which is administered to the mammal is effective to reduce or inhibit the function of the viral sequence in the cells of the mammal.

While not wishing to be bound by theory, once the RNA molecule is delivered to or produced in a cell infected by the virus, the exogenous RNA molecule reduces or inhibits (i.e. turns off) the homologous viral sequence and is itself inhibited, so that the function of the viral sequence is reduced or inhibited. As demonstrated in the examples below, the inhibition of function effect is transferred from the mammalian cell which receives the exogenous RNA molecule to other mammalian cells in the subject which have not directly been provided with the exogenous RNA molecule. It is presently theorized that this results occurs on the level of RNA degradation.

Thus, this method can be used to treat mammalian subjects already infected with a virus, such as HIV, in order to shut down or inhibit a viral gene function essential to virus replication and/or pathogenesis, such as HIV gag. Alternatively, this method can be employed to inhibit the functions of viruses which exist in mammals as latent viruses, e.g., *Varicella zoster* virus, and are the causative agents of the disease known as shingles. Similarly, diseases such as atherosclerosis, ulcers, chronic fatigue syndrome, and autoimmune disorders, recurrences of HSV-1 and HSV-2, HPV persistent infection, e.g., genital warts, and chronic HBV infection among others, which have been shown to be caused, at least in part, by viruses, bacteria or another pathogen, can be treated according to this method by targeting certain viral polynucleotide sequences essential to viral replication and/or pathogenesis in the mammalian subject.

In still another embodiment of this invention, the compositions described above can be employed in a method to prevent viral infection in a mammal. When the method described above, i.e., administering a composition described above in an amount effective to reduce or inhibit the function of the essential target viral polynucleotide sequence to a mammal, is administered prior to exposure of the mammal to the virus, it is expected that the exogenous RNA molecule remains in the

mammal and work to inhibit any homologous viral sequence which presents itself to the mammal thereafter. Thus, the compositions of the present invention may be used to inhibit or reduce the function of a viral polynucleotide sequence for vaccine use.

Still an analogous embodiment of the above "anti-viral" methods of the invention includes a method for treatment or prophylaxis of a virally induced cancer in a mammal. Such cancers include HPV E6/E7 virus-induced cervical carcinoma, HTLV-induced cancer, and EBV induced cancers, such as Burkitts lymphoma, among others. This method is accomplished by administering to the mammal a composition as described above in which the target polynucleotide is a sequence encoding a tumor antigen or functional fragment thereof, or a non-expressed regulatory sequence, which antigen or sequence function is required for the maintenance of the tumor in the mammal. Among such sequences are included, without limitation, HPV16 E6 and E7 sequences and HPV 18 E6 and E7 sequences. Others may readily be selected by one of skill in the art. The composition is administered in an amount effective to reduce or inhibit the function of the antigen in the mammal, and preferably employs the composition components, dosages and routes of administration as described above. The molecular mechanism underlying this method is the same as that described above.

In another embodiment of the invention, the compositions of this invention can be employed in a method for the treatment or prophylaxis of infection of a mammal by a non-viral pathogen, either intracellular or extracellular. As used herein, the term "intracellular pathogen" is meant to refer to a virus, bacteria, protozoan or other pathogenic organism that, for at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogenic proteins. Intracellular pathogens which infect cells which include a stage in the life cycle where they are intracellular pathogens include, without limitation, Listeria, Chlamydia, Leishmania, Brucella, Mycobacteria, Shigella, and as well as Plasmodia, e.g., the causative agent of malaria, *P. falciparum*. Extracellular pathogens are those which replicate and/or propagate outside of the mammalian cell, e.g., Gonorrhoeae, and Borrellia, among others. According to this embodiment, such infection by an pathogen may be treated or possibly prevented by administering to a mammalian

subject, either already infected or anticipating exposure to the pathogen, with a composition as described above with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier. In this case, the RNA molecule of the composition has a polynucleotide sequence which is substantially homologous to a target polynucleotide sequence of the pathogen that is necessary for replication and/or pathogenesis of the pathogen in an infected mammal or mammalian cell. As above, the amount of the composition administered is an amount effective to reduce or inhibit the function of the pathogenic sequence in the mammal. The dosages, timing, routes of administration and the like are as described above.

One of skill in the art, given this disclosure can readily select viral families and genera, or pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites, for which therapeutic or prophylactic compositions according to the present invention can be made. See, e.g., the tables of such pathogens in general immunology texts and in U. S. Patent No. 5,593,972, incorporated by reference herein.

The compositions of this invention and possibly protein-encoding molecules of the prior art may also be employed in another novel method of this invention. Such compositions are also useful in the treatment of certain non-pathogenic diseases or disorders of mammals, such as certain cancers or inherited disorders. Among conditions particularly susceptible to treatment or prophylaxis according to this invention are those conditions which are characterized by the presence of an aberrant mammalian polynucleotide sequence, the function of which is necessary to the initiation or progression of the disorder, but can be inhibited without causing harm or otherwise unduly adversely impacting the health of the mammal. In other words, a characteristic of a disorder suitable for this treatment is that the mammal can survive without the function of the gene, or can survive if the function of the gene was substantially reduced. In such cases, the function of the aberrant or abnormal polynucleotide sequence can be replaced exogenously by therapy. In another case, the disease can be caused by the presence or function of an abnormal polynucleotide

sequence or gene in a mammal, where the mammal also possesses a normal copy of the polynucleotide sequence or gene, and wherein the differences between the abnormal gene and the normal gene are differences in nucleotide sequence. In such cases, inhibition of the function of the abnormal polynucleotide sequence by the method of this invention is likely to permit the normal polynucleotide sequence to function, without exogenous treatment.

Thus, in one embodiment, a method of treatment or prophylaxis of a cancer in a mammal involves administering to the mammal a composition of this invention in which the target polynucleotide sequence is an abnormal cancer-causing polynucleotide sequence or gene in a mammal. The composition of this invention is administered in an amount effective to reduce or inhibit the function of the abnormal sequence in the mammal. As described above, the composition can contain an optional second agent that facilitates polynucleotide uptake in a cell, and a pharmaceutically acceptable carrier, and be administered in dosages, regimens and by routes as described above.

Mammalian cancers which are characterized by the presence of abnormal and normal polynucleotide sequences include chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL), where the abnormal sequence is a fusion of two normal genes, i.e., *bcr-abl*. See, e.g., the description of these cancers in International Patent Publication No. WO94/13793, published June 23, 1994, and incorporated herein by reference for a description of these diseases. In such cancers or diseases, such as CML, the afflicted mammal also possesses a normal copy of the polynucleotide sequence or gene, and the differences between the abnormal and normal sequences or genes are differences in nucleotide sequence. For example, for CML, the abnormal sequence is the bcr-abl fusion, while the normal sequence is bcr and abl. Thus, the method above can be employed with the target polynucleotide sequence being the sequence which spans the fusion. A method of treatment or prophylaxis of such a cancer in a mammal comprises administering to the mammal a composition of this invention wherein the target polynucleotide is a polynucleotide sequence of an abnormal cancer-causing gene in a mammal which also possesses a

normal copy of the gene, and wherein the differences between the abnormal gene and the normal gene are differences in polynucleotide sequence. The composition is administered as above, with an optional second agent that facilitates polynucleotide uptake in a cell, and in a pharmaceutically acceptable carrier and in an amount effective to reduce or inhibit the function of the abnormal sequence in the mammal.

The present invention thus encompasses methods for evoking the above-described molecular mechanism for treating any disease or disorder in a mammal characterized by expression of an undesirable polynucleotide product or polynucleotide mediated function not found in a healthy mammal by use of a composition which can deliver to the cells of the mammal the partially double-stranded RNA molecule substantially homologous to the target polynucleotide sequence which expresses or mediates the undesired product or function, in an amount effective to reduce or inhibit the function of that polynucleotide in the cells of the mammal. Provided that the RNA molecule is sufficiently non-homologous to essential mammalian polynucleotide sequences, so that it does not inhibit the function of those essential sequences, this method can be clearly seen to have many therapeutic and prophylactic uses. One of skill in the art can readily select disorders described above, and can also readily select target polynucleotide sequences against which the compositions of the present invention are directed.

D. Other Methods of The Present Invention

The compositions described above, and the general methods of using these compositions to inhibit or reduce the function of a target polynucleotide sequence, can also be applied to a variety of research, and *in vitro* applications. For example, the method of this invention can be applied to research to determine the function of a selected polynucleotide sequence in a cell line, or a mammalian laboratory animal, by administering to that cell in tissue culture or that animal *in vivo* a composition of the invention wherein the RNA molecule polynucleotide sequence is substantially homologous to the selected sequence and preferably substantially non-homologous to

other polynucleotide sequences in the animal. The inhibition of the function of that target sequence permits study of its influence on the animal's biology and physiology.

Similarly, application of this method can be used to make cell lines of mammalian, bacterial, yeast, fungal, insect and other origins defective in selected pathways by "silencing" a selected functional sequence, such as an enzymatic sequence, a protein expressing sequence, or regulatory sequences necessary to the expression thereof. Such manipulated cells may be employed in conventional assays or drug screening assays, etc.

In an analogous method, a "knock-out" laboratory animal can be prepared by altering the dosage of administration sufficient to permanently shut off the function of a selected gene. Thus, the method of the present invention in delivering an RNA molecule with a polynucleotide sequence sufficiently homologous to the sequence selected to be "knocked out" in the laboratory animal as described above provides a simpler technique for developing "knock-out" mice and other laboratory animals useful for pharmaceutical and genetic research.

Still other research methods for use of the compositions and methods of this invention include the preparation of mutants of microorganisms, both eukaryotic and prokaryotic, for use as research agents or as industrial production strains for the microbial production of desired proteins. Still other uses are expected to be obvious to the person of skill in the art given the teachings herein.

The following examples illustrate methods for preparing the compositions and using the compositions of this invention to reduce or inhibit target polynucleotide sequences. These examples which employ as the agent of the composition, double stranded RNA molecules made by *in vitro* synthesis and target polynucleotide sequences of HIV gag or HSV gD2 merely illustrate embodiments of this invention. It is understood by one of skill in the art, that other selections for the various agents of the compositions, and identity of the target polynucleotide sequences may be readily selected as taught by this specification. These examples are illustrative only and do not limit the scope of the invention.

HIV(HXB2), Genbank Accession number K03455 [see also, L. Ratner *et al.*, AIDS Res. Hum. Retroviruses, 3(1):57-69 (1987)]. The Forward gag primer maps to coordinates 901-924 and this sequence follows the T7 promoter in the T7 Forward gag primer. The Reverse gag primer maps to coordinates 1476-1500 and follows the T7 promoter in the T7 Reverse gag primer.

To generate a composition of this invention where the agent is single-stranded sense RNA, a T7 promoter is located at the 5' end of the forward PCR primer. The PCR primers used to generate the DNA template that encodes the ss sense RNA, written 5' to 3' with the top strand of the T7 promoter underlined, are the T7 forward gag primer [SEQ ID NO: 1]:

5'GTAATACGACTCACTATAGGGCGGCAGGGAGCTAGAACGATTCGCAG 3'

and the Reverse gag primer [SEQ ID NO: 2]:

5'CTGCTATGTCACTTCCCCTTGGTTC 3'

To generate a composition where the agent is a single stranded anti-sense RNA molecule, the T7 promoter is located at the 5' end of the reverse PCR primer. These primers are the T7 Reverse gag primer [SEQ ID NO: 3]:

5' GTAATACGACTCACTATAGGGCGCTGCTATGTCACTTCCCCTTGGTTC 3'

and the Forward gag primer [SEQ ID NO: 4]:

5' GCAGGGAGCTAGAACGATTCGCAG 3'.

Both types of PCR products described above are included in the T7 transcription reaction to generate a composition where the agent is double-stranded RNA molecule. Alternatively, an agent of the composition according to this invention is prepared by mixing together sense and anti-sense RNA after transcription.

As a control, similarly sized sense RNA, antisense RNA, and dsRNA molecules are derived from the gD gene of a Herpes Simplex Virus, type 2 genome are generated by the same PCR and T7 transcription techniques. The coordinates of the PCR primers for HSV gD are derived from the map of GenBank Accession number K01408, HSVgD2 gene. The Forward gD primer maps to coordinates 313-336; this sequence follows the T7 promoter in the T7 Forward gD primer. The

Reverse gD primer maps to coordinates 849-872, and follows the T7 promoter in the T7 Reverse gD primer. The primer sets used to generate these control molecules were:

T7 forward gD primer [SEQ ID NO: 5]:

5' GTAATACGACTCACTATAGGGCGGTCGCGGTGGGACTCCGCGTCGTC 3'

and

Forward gD primer [SEQ ID NO: 6]: 5' GTCGCGGTGGGACTCCGCGTCGTC 3';

and

T7 reverse gD primer [SEQ ID NO: 7]:

5' GTAATACGACTCACTATAGGGCGGTGATCTCCGTCCAGTCGTTTATC 3'

and

Reverse gD primer [SEQ ID NO: 8]: 5' GTGATCTCCGTCCAGTCGTTTATC 3'.

These RNA molecules of the invention and the above-described control molecules are assayed with the RD and COS7 cell lines as follows:

Between 5-6 x10^5 cells/well in six-well plates are cultured to about 80-90% confluence, and are transfected with 2-3 μg of a selected RNA molecule or control molecule, using 10 μl lipofectamine (Gibco-BRL) as a transfecting agent. Transfected cells are incubated for times ranging between 1 to 17 hours. Another cell culture was transfected with doses of RNA ranging between 1 μg to 500 μgs, delivered with no known transfecting agent and incubated on the cells from 0.5 minutes to about two days. For example, one group of cells is transfected with the sense gag RNA, another with the antisense gag RNA, another with ds gag RNA, another with sense gD RNA control, another with antisense gD RNA control, and another with ds gD RNA control. Also additional negative controls are cells which receive no RNA molecules.

The cells are cultured at 37°C and monitored for p24 synthesis over the course of several weeks. The cells are assayed three times per week after two days post-administration of RNA, both by measuring p24 in the media of cells using the p24 ELISA assay kit (Coulter Corp) and by immunostaining fixed cells for p24 using a rabbit polyclonal anti-p24 sera (Intracell Corp.) and anti-rabbit IgG that is FITC-conjugated (Sigma).

According to the present invention, none of the gD RNA molecules demonstrate the ability to retard or inhibit p24 synthesis. However, according to the invention the ds gag RNA inhibits or down regulates p24 synthesis. The sense and antisense RNA molecules are expected to cause only a modest, if any, inhibitory effect on p24 synthesis, unless these RNAs were able to form some degree of double strandedness.

EXAMPLE 2: DETERMINATION OF THE EXTENT OF REDUCTION OF P24 SYNTHESIS FROM ONE CELL CULTURE TO ANOTHER

To demonstrate that the down-regulated signal can be transmitted to cells which have not been down-regulated, this example demonstrates that the reduction/inhibition effect (i.e., inhibition or reduction of p24 synthesis) is transmitted to cells in culture that are not transfected by the agent.

A. Co-Culture of COS 7 and RD cells

Cells from the cultures of Example 1 which demonstrate reduction of p24 synthesis are co-cultured with control cells of cells that have not previously been incubated with any RNA molecule, and are, in fact, synthesizing p24 at wild-type levels. According to the present invention, the previously transfected cells can transfer the target polynucleotide function inhibition to non-transfected cells, and the control cells in the co-culture are characterized by a reduction in synthesis of p24.

In order to distinguish control cells from the previously transfected cells in the culture, a first protocol is followed: The COS 7 cells of Example 1 which demonstrate inhibition of p24 synthesis are co-cultured with non-transfected RD cells expressing p24 at wildtype levels at various ratios of cell types, e.g., the ratios range from 1/1000 to 1/10 (COS 7/RD) to a total of 6 - 7 x10^5 cells in 6 well plates. After 2 days of culture under the conditions specified in Example 1, the RD cells in the cultures are examined for p24 synthesis. The cells are examined about 3 times per week for 3 weeks.

p24 synthesis is assayed by two methods. In the first method, the media from the co-cultured cells is assayed for p24 using the p24 ELISA assay

(Coulter). In the second method, cells are immunostained for p24 using rabbit polyclonal sera (Intracell Corp.) against p24 and anti-rabbit IgG conjugated to FITC. Because COS 7 and RD cells are distinguishable by morphology, a loss of stain in the RD cells can be readily distinguished from the COS 7 cells. Because COS 7 cells express T Antigen while RD cells do not, the co-cultured cells are also stained for T Ag using mouse monoclonal sera against SV40 T antigen (Pharmagen Corp.) and anti-mouse IgG conjugated to r-phycoerythrin (PE). Only the COS 7 cells stain under these conditions. The cell staining is determined by fluorescence microscopy or by FLOW cytometry.

The inhibition of p24 function in RD cells in coculture is demonstrated by comparison to a control culture containing only the RD cells by a loss of FITC stain in the co-cultured RD cells. RD cells in the coculture that are not stained with FITC or PE are evidence of reduction or inhibition of the p24 synthesis function of the p24 target polynucleotide by the RNA molecules (particularly the ds RNA molecules) of Example 1.

B. Cultures of transfected RD cells with non-transfected RD cells

In a second protocol, the transfected RD cells of Example 1, which demonstrate reduced p24 production are co-cultured with non-transfected RD cells which are engineered to contain an integrated hygromycin resistance gene and express normal levels of p24 using different ratios of cells, with ratios ranging from 1/1000 to 1/10 (RD/control RD) to a total cell number of 6 - 7 x10^5 in a 6 well plate. Hygromycin-resistant RD cells are made as follows: RD cells (5-6 x10^5 cells) are cultured to 80-90% confluence in a six-well plate and are transfected with 2.5 μg of the Nru 1-Sal 1 fragment of pCEP4 (Invitrogen Corp.) that contains the hygromycin resistance gene under the control of a thymidine kinase (TK) promoter. Transfections are done using the transfecting agent, lipofectamine (Gibco BRL). Two days following transfection, the cells are incubated in the presence of 400 μg/ml hygromycin. Resistant cells are clonally expanded. One or more of the clonally expanded cell lines are used as the control in the experiment.

From 1 day to several weeks after co-culture under the conditions specified in Example 1, replicate co-cultures are incubated with 400 µg/ml hygromycin. This concentration of hygromycin kills the RD cells that are not hygromycin resistant, leaving only the control hygromycin resistant RD cells. The remaining resistant cells are derived from the control cells. P24 levels are measured directly from the control cells, for example using the ELISA of Example 1 as well as by immunostaining as above described.

According to the present invention, inhibition of p24 production is revealed in at least a subset of the control cells.

EXAMPLE 3: *IN VIVO* INHIBITION OF ENDOGENOUS INTERLEUKIN-12 PRODUCTION BY THE METHOD OF THIS INVENTION

A. Design of RNA molecules as Compositions of the Invention

All RNA molecules in this experiment are close to 600 nts in length, and all RNA molecules are designed to be incapable of producing the p40 chain of IL-12. The molecules have no cap and no poly-A sequence; the native initiation codon is not present, and the RNA does not encode the full-length product. The following RNA molecules are designed:

(1) a single-stranded (ss) sense RNA polynucleotide sequence homologous to IL-12 p40 murine messenger RNA (mRNA);

(2) a ss anti-sense RNA polynucleotide sequence complementary to IL-12 p40 murine mRNA,

(3) a double-stranded (ds) RNA molecule comprised of both sense and anti-sense p40 IL-12 murine mRNA polynucleotide sequences,

(4) a ss sense RNA polynucleotide sequence homologous to IL-12 p40 murine heterogeneous RNA (hnRNA),

(5) a ss anti-sense RNA polynucleotide sequence complementary to IL-12 p40 murine hnRNA,

(6) a ds RNA molecule comprised of the sense and anti-sense IL-12 p40 murine hnRNA polynucleotide sequences,

15. The composition according to claim 13 wherein said single stranded RNA anti-sense strand folds back upon itself to become partially double stranded.

16. The composition according to claim 1, wherein said agent is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence, said single stranded RNA sequence having the ability to become double-stranded.

17. The composition according to claim 1 wherein said agent is a circular RNA molecule that forms a rod structure.

18. The composition according to claim 8 wherein said agent is a double stranded DNA molecule encoding said RNA molecule.

19. The composition according to claim 18 wherein said DNA encodes a double stranded RNA.

20. The composition according to claim 18 wherein said DNA encodes a single stranded RNA sense strand.

21. The composition according to claim 20 wherein said DNA encodes a single stranded RNA sense strand that forms a hairpin at one or both termini or intermediate therebetween.

22. The composition according to claim 20 wherein said DNA encodes a single stranded RNA sense strand that folds back upon itself to become partially double stranded.

23. The composition according to claim 18 wherein said DNA encodes a single stranded RNA anti-sense strand.

24. The composition according to claim 23 wherein said DNA encodes a single stranded RNA anti-sense strand that forms a hairpin at one or both termini or intermediate therebetween.

25. The composition according to claim 23 wherein said DNA encodes a single stranded RNA anti-sense strand that folds back upon itself to become partially double stranded.

26. The composition according to claim 18 wherein said DNA encodes a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence, said single stranded RNA sequence having the ability to become double-stranded.

27. The composition according to claim 18 wherein said DNA encodes a circular RNA molecule that forms a rod structure.

28. The composition according to claim 1, wherein said agent is a plasmid.

29. The composition according to claim 1, wherein said agent comprises a first DNA plasmid encoding a single stranded RNA sense polynucleotide sequence and a second DNA plasmid encoding a single stranded RNA anti-sense polynucleotide sequence, wherein said sense and anti-sense RNA sequences have the ability to base-pair and become double-stranded.

30. The composition according to claim 28, wherein said plasmid comprises bacterial sequences.

31. The composition according to claim 1, wherein said agent is a recombinant bacterium.

32. The composition according to claim 1, wherein said agent is a recombinant virus.

33. The composition according to claim 1, wherein said agent is a donor cell transfected *in vitro* with the molecule described in any of claims 2 through 32.

34. The composition according to any of claims 30-32, wherein said agent is selected from the group consisting of a living recombinant virus or bacteria or cell, a dead virus or bacteria or cell, or an inactivated virus or bacteria or cell.

35. The composition according to claim 1, wherein said agent lacks a poly-adenylation sequence.

36. The composition according to claim 1, wherein said RNA molecule is not translated.

37. The composition according to claim 1, wherein said agent lacks a Kozak region.

38. The composition according to claim 1, wherein said agent lacks an initiating methionine codon.

39. The composition according to claim 1 wherein said RNA molecule lacks a cap structure.

40. The composition according to claim 1 wherein said agent lacks signals for protein synthesis.

41. The composition according to claim 1, comprising a mixture of different said agents.

42. The composition according to claim 1 wherein said target polynucleotide sequence is a virus polynucleotide sequence necessary for replication and/or pathogenesis of said virus in an infected mammalian cell.

43. The composition according to claim 42, wherein said virus is selected from the group consisting of a DNA virus and a virus that has an intermediary DNA stage.

44. The composition according to claim 43, wherein said virus is selected from the group consisting of Retrovirus, Herpesvirus, Hepadenovirus, Poxvirus, Parvovirus, Papillomavirus, and Papovavirus.

45. The composition according to claim 44, wherein said virus is selected from the group consisting of HIV, HBV, HSV, CMV, HPV, HTLV and EBV.

46. The composition according to claim 1, wherein said target polynucleotide sequence is a tumor antigen or functional fragment thereof or a regulatory sequence of a virus-induced cancer, which antigen or sequence is required for the maintenance of said tumor in said mammal.

47. The composition according to claim 46, wherein said cancer is selected from the group consisting of HPV E6/E7 virus-induced cervical carcinoma, HTLV-induced cancer and EBV induced cancer.

48. The composition according to claim 1, wherein said target polynucleotide sequence is a polynucleotide sequence of an intracellular or extracellular pathogen necessary for replication and/or pathogenesis of said pathogen in an infected mammalian cell.

49. The composition according to claim 1 wherein said target polynucleotide sequence is a polynucleotide sequence of an abnormal cancer-causing sequence in a mammal which also possesses a normal copy of said sequence, and wherein the differences between the abnormal and the normal sequences are differences in polynucleotides.

50. The composition according to claim 49 wherein said abnormal sequence is a fusion of two normal genes.

51. The composition according to claim 50 wherein said target polynucleotide is the polynucleotide sequence spanning said fusion.

52. A pharmaceutical composition comprising a composition of any of claims 1-51, and an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier.

53. The composition according to claim 52, wherein said second agent is selected from the group consisting of a local anaesthetic, a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation, a branched, three-dimensional polycation, a carbohydrate, a cationic amphiphile, a detergent, a benzylammonium surfactant, or another compound that facilitates polynucleotide transfer to cells.

54. The composition according to claim 53 wherein said second agent is bupivacaine.

<210> 18
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: forward IL-12 cDNA

<400> 18
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<210> 19
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: reverse IL-12 cDNA

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<210> 20
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: T7 reverse IL-12 cDNA

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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES

(57) Abstract: A therapeutic composition for inhibiting the function of a target polynucleotide sequence in a mammalian cell includes an agent that provides to a mammalian cell an at least partially double-stranded RNA molecule comprising a polynucleotide sequence of at least about 200 nucleotides in length, said polynucleotide sequence being substantially homologous to a target polynucleotide sequence. This RNA molecule desirably does not produce a functional protein. The agents useful in the composition can be RNA molecules made by enzymatic synthetic methods or chemical synthetic methods*in vitro*; or made in recombinant cultures of microorganisms and isolated therefrom, or alternatively, can be capable of generating the desired RNA molecule *in vivo* after delivery to the mammalian cell. In methods of treatment of prophylaxis of virus infections, other pathogenic infections or certain cancers, these compositions are administered in amounts effective to reduce or inhibit the function of the target polynucleotide sequence, which can be of pathogenic origin or produced in response to a tumor or other cancer, among other sources.

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 00/10555

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/11 C12N15/63 C12N15/70 C12N15/86 C07K14/01
C07K14/08 A61K48/00 A61K38/16 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MONTGOMERY M K ET AL: "Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression" TRENDS IN GENETICS,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 14, no. 7, 1 July 1998 (1998-07-01), pages 255-258, XP004124680 ISSN: 0168-9525 the whole document ---	1
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2000	15/11/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mateo Rosell, A.M.

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Interr ıal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 05770 A (ROTHBARTH KARSTEN ;JOSWIG GABY (DE); WERNER DIETER (DE); SCHUBERT) 12 February 1998 (1998-02-12) cited in the application the whole document	1,6,8, 18,20, 23-25, 28,30, 31, 33-40, 49,59, 60,64
X	WO 97 34638 A (UNIV CALIFORNIA) 25 September 1997 (1997-09-25) page 3, line 5 -page 4, line 28; examples 1,2	1,6,8, 13,28, 32-34, 42-45, 48,55-67
X	PLAYER MARK R ET AL: "Targeting HIV mRNA for degradation: 2,5-A antisense chimeras as potential chemotherapeutic agents for AIDS." NUCLEOSIDES & NUCLEOTIDES, vol. 16, no. 7-9, July 1997 (1997-07), pages 1221-1222, XP000952972 ISSN: 0732-8311 the whole document	1,42-45
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A	US 4 891 315 A (WEIS JOHN H ET AL) 2 January 1990 (1990-01-02) the whole document	1,42,45
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Intern al Application No
PCT/US 00/10555

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YUKIKO TONE ET AL: "STRUCTURE AND CHROMOSOMAL LOCATION OF THE MOUSE INTERLEUKIN-12 P35 AND P40 SUBUNIT GENES" EUROPEAN JOURNAL OF IMMUNOLOGY,DE,WEINHEIM, vol. 26, no. 6, 1 June 1996 (1996-06-01), pages 1222-1227, XP000612644 ISSN: 0014-2980 cited in the application the whole document ---	1
A	JAEGER J A ET AL: "IMPROVED PREDICTIONS OF SECONDARY STRUCTURES FOR RNA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 86, no. 20, 1989, pages 7706-7710, XP000953154 1989 ISSN: 0027-8424 cited in the application the whole document ---	2
A	WATERHOUSE ET AL: "Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,NATIONAL ACADEMY OF SCIENCE. WASHINGTON,US, vol. 95, November 1998 (1998-11), pages 13959-13964, XP002114472 ISSN: 0027-8424 the whole document ---	1,42
A	NELLEN W ET AL: "WHAT MAKES AN MRNA ANTI-SENSE-ITIVE?" TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 18, no. 11, 1 November 1993 (1993-11-01), pages 419-423, XP002049385 ISSN: 0968-0004 the whole document ---	1
A	FIRE A ET AL: "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 391, 19 February 1998 (1998-02-19), pages 806-811, XP002095876 ISSN: 0028-0836 the whole document --- -/--	1

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